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In Vitro Studies on Clonal Growth of Chondrocytes in Thanatophoric Dysplasia

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Thanatophoric dysplasia (TD) is characterized by a disorganized growth plate with markedly reduced proliferative and hypertrophic cartilage zones. Therefore, we studied *in vitro* the proliferation rates of articular chondrocytes from five TD patients and age-matched controls in response to bFGF, IGF-I, IGF-II, and TGF-β1. In human fetal controls bFGF was the most potent growth factor. Clonal growth of articular chondrocytes in response to bFGF was reduced in two of five TD patients and slightly below the range of controls in a third case. Stimulation of chondrocyte proliferation by IGF I and II was reduced in the patient whose response to bFGF was most markedly impaired. The effect of TGF-β1 ranged from normal to slightly elevated values in TD fetuses. These results indicate heterogeneity of the underlying defects in TD. Low proliferative responses of chondrocytes to bFGF and IGF-I/II are likely to play a key role in the pathogenesis of some cases. In two of five patients studied, the mechanisms of bFGF and IGF-signal transduction are candidates for the primary molecular defect.

KEY WORDS: thanatophoric dysplasia, chondrocyte, clonal growth, IGF-I, IGF-II, TGF-b, bFGF

INTRODUCTION

Thanatophoric dysplasia (TD) is the most common human lethal skeletal dysplasia [Connor et al., 1985]. Patients are born with short limbs, normal length of the trunk, narrow thorax, a disproportionately large head circumference, a depressed nose, and prominent forehead [Rimoin, 1975; Yang et al., 1976]. Most patients are stillborn or die soon after birth because of respiratory failure due to lung hypoplasia. However, some patients may survive for several months [Stensvold et al., 1986].

Radiographs show short and bowed tubular bones with flared metaphyses, short ribs, flat vertebral bodies with central impression in lateral and anteroposterior X-rays and short, broad iliac wings [Spranger and Maroteaux, 1990]. Recently, these patients have been classified as TD type 1 in contrast to TD type 2 which is characterized by an additional cloverleaf skull. In this type of TD the extracranial skeletal abnormalities are less severe compared to TD type 1 [Yang et al., 1976; Spranger and Maroteaux, 1990].

Histological changes of TD are well characterized by a severe disturbance of the growth plate. In TD chondrocyte proliferation and hypertrophic zones are markedly reduced [Sillence et al., 1979]. Studies on native cartilage excluded major structural defects in several components of the extracellular matrix like collagen II, proteoglycan link proteins and cartilage oligomeric matrix protein [Horton et al., 1989; Stanescu et al., 1991, 1994].

Recently, in achondroplasia and hypochondroplasia as members of the same family of skeletal dysplasias [Spranger, 1985] as well as in TD mutations of the fibroblast growth factor receptor 3 (FGFR3) have been described [Shiang et al., 1994; Rousseau et al., 1994; Bellus et al., 1995; Tavormina et al., 1995; Rousseau et al., 1995]. We studied proliferative signal transduction on the cell-biological level and determined clonal growth of articular chondrocytes from 5 patients with TD type I and age-matched controls in response to bFGF, IGF-I, IGF-II, and TGF-β1.

PATIENTS AND METHODS

Tissue Sampling

Samples of articular cartilage from the distal femur were obtained at autopsy from five patients with characteristics of thanatophoric dysplasia type 1 (without cloverleaf skull). All patients died perinatally. Diagnosis was confirmed by typical radiographs and routine

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Abbreviations: TD–thanatophoric dysplasia; IGF–insulin-like growth factor; TGF–transforming growth factor; FCS–fetal calf cerum; bFGF–basic fibroblast growth factor.

TABLE I. Clinical and Radiological Data of the Patients With Thanatophoric Dysplasia (TD)

	Gestational age (weeks)	Bell-shaped thorax	"Telephone-receiver" long bones	Dysplastic os ileum	CNS malformations
TD 1	18	−	+	(+)	Hypoplastic cerebellum Anomalous gyration of occipital lobes
TD 2	32	+	+	+	Hypoplastic cerebellum Hypoplastic tractus of medulla and cerebrum
TD 3	37	+	+	(+)	No major malformation (not examined in detail)
TD 4	24	+	+	+	Micropolygyria of hippocampus Status marmoratus of basal ganglia
TD 5	33	+	+	+	Hypoplastic CNS (inhomogenous, global)

histology of the growth plate (HE-, van Gieson's-, and PAS-staining). Clinical and radiological data are summarized in Table I. Gestational ages of the TD patients were 18, 24, 32, 33, and 37 weeks. In parallel, five fetal controls (15, 18, 19, 21, and 30 weeks of gestation) who died of diseases not related to the skeletal system were studied. The studies were performed with approval of the local ethics committee.

Cell Cultures

Tissue samples of articular cartilage were carefully cleaned of adherent tissue and dissected into small pieces. Isolation and cell culture conditions for chondrocytes were performed as described by Vetter et al. [1986]. Briefly, the pieces of cartilage were digested with trypsin (500 U/ml, Seromed-Biochrom, Berlin, Germany) and collagenase (CLS II, 300 U/ml, Seromed-Biochrom, Berlin, Germany) for 30 minutes and after a washing step with collagenase (300 U/ml) alone for 16 hours, both at 37°C. After centrifugation the cell pellet was resuspended in Ham's F-10 medium supplemented with 10% FCS, 200 mmol/l L-glutamin, ascorbate, and MEM vitamins (Seromed-Biochrom, Berlin, Germany). Cultivation of chondrocytes was performed under 5% O_2, 5% CO_2, and with 95% relative humidity.

Proliferation Studies

Clonal growth of chondrocytes was determined as described by Vetter et al. [1986]. Freshly isolated chondrocytes were seeded in BM Whissler Medium (Boehringer Mannheim, Germany) supplemented with 5% heat-inactivated FCS, 40 μg/ml netilmicin, 0.1 mol/l mercaptoethanol, MEM-vitamins, and 0.8% methylcellulose (Fluka, Buchs, Switzerland) with or without various concentrations of human bFGF (0.3/1.25/12.5 ng/ml, Sigma, Deisenhofen, Germany), human IGF-I (0.3/1.25/12.5 ng/ml, Amersham, Braunschweig, Germany), human IGF-II (0.3/1.25/12.5 ng/ml, kindly supplied by Dr. Humbel, Institute for Biochemistry, University of Zürich), or human TGF-β1 (0.3/1.25 ng/ml, Peninsula, CA). After 12 days the numbers of colonies were counted and the results were expressed as per cent of the basal value obtained with 5% heat-inactivated FCS alone. All experiments were performed in triplicate.

RESULTS

Histological Evaluation

The routine morphological analysis showed typical pathohistologic changes of the osteo-chondrous junction in TD fetuses. These comprised a strong reduction of the chondrocyte proliferation and hypertrophy zones, along with normal appearance of the resting cartilage, multiple perforating vascular channels crossing the growth plate, connective tissue interposition between cartilage and bone and partly increased periosteal ossification. The severity of these histopathologic changes was semiquantitatively estimated. The pathomorphologic data are summarized in Table II.

TABLE II. Histopathologic Features of the Patients With Thanatophoric Dysplasia (TD)

	Resting cartilage	Proliferative/ hypertrophic cartilage	Connective tissue interposition	Perforating vascular channals	Periosteal ossification	Structure of primary spongiosa
TD 1	Normo-cellular	Slightly reduced	Focally	Slightly increased	Regular	Adequate
TD 2	Normo-cellular	Significantly reduced	Widely	Significantly increased	Increased	Adequate
TD 3	Normo-cellular	Significantly reduced	n.d[a]	n.d	n.d	Adequate
TD 4	Normo-cellular	Significantly reduced	Marginally	Slightly increased	Increased	Adequate
TD 5	Normo-cellular	Significantly reduced	Marginally	Significantly increased	Increased	Adequate

[a] n.d., not determined.

TABLE III. Clonal Growth of Articular Chondrocytes From Fetal Controls and Patients With Thanatophoric Dysplasia (TD) in Response to bFGF (% of Basal Value)

bFGF	0.3 ng/ml (%)	1.25 ng/ml (%)	12.5 ng/ml (%)
Controls (M ± SD, n = 4)	343 ± 70	484 ± 155	674 ± 213
Range	250–417	345–704	403–913
TD 1[a]	135 ± 23	211 ± 7	361 ± 25
TD 2[a]	147 ± 2	156 ± 9	221 ± 23
TD 3[a]	197 ± 3	344 ± 30	400 ± 43
TD 4[a]	397 ± 101	508 ± 34	1164 ± 177
TD 5[a]	473 ± 15	530 ± 62	1647 ± 355

[a]M ± SD of triplicate determinations.

Clonal Growth of Chondrocytes

Clonal growth of articular chondrocytes was studied in response to various concentrations of bFGF, IGF-I, IGF-II, and TGF-β1. The absolute range of colony incidence under basal conditions did not differ between controls and TD patients regardless of the gestational ages (10–120 colonies out of 1,000 cells in controls and 14–140 colonies out of 1,000 cells in TD fetuses). The mean standard deviation of triplicate determinations was 8.9% of the mean in controls (n = 52) and TD patients (n = 55). In fetal controls all growth factors studied stimulated clonal growth of articular chondrocytes. However, stimulation of chondrocyte proliferation was highest with bFGF at all concentrations (Tables III–VI).

Articular chondrocytes from two fetuses with TD (patients 1 and 2) showed a markedly reduced stimulation of clonal growth with all concentrations of bFGF. Patient 3 had values slightly below the range of controls. The remaining two TD patients had a normal or even elevated colony incidence in response to bFGF (Table III). Stimulation of clonal growth with IGF-I and IGF-II was reduced in one and in the lower range of controls in three patients (Tables IV, V). The colony incidence under treatment with TGF-β1 ranged from normal to slightly elevated values in TD fetuses (Table VI).

DISCUSSION

The histological analysis of our TD patients confirmed previous reports of a normal resting cartilage and a severely disturbed organization of the cartilaginous growth plate [Sillence et al., 1979; Ornoy et al., 1985]. In particular, the reduction of the proliferative and hypertrophic zone suggested a disturbed regulation of chondrocyte proliferation and/or maturation. This assumption is now confirmed by our *in vitro* results showing that the response of chondrocyte growth to supplementation with bFGF/IGF-I/II was reduced in some of the TD cases studied here. In addition, our data provide some significant evidence that the underlying molecular defects in TD may be heterogeneous. These results are in agreement with recent publications from Tavormina et al. [1995] and Rousseau et al. [1995] who identified mutations of the FGFR3 in patients with TD type I and II. While in achondroplasia mutations of the transmembrane domain of the FGFR3 were found [Shiang et al., 1994; Rousseau et al., 1994; Stoilov et al., 1995; Bellus et al., 1995] in TD type I mutations of the extracellular part and in TD type II and hypochondroplasia mutations of the intracellular tyrosin kinase domains of the FGFR3 could be identified [Tavormina et al., 1995; Rousseau et al., 1995; Bellus et al., 1995]. However, Tavormina et al. [1995] could not detect FGFR3 mutations in 45% of their patients with TD type I confirming the assumption of heterogeneity of the primary defect.

IGF-I/II and TGF-β1 have previously been shown to stimulate clonal growth of normal human fetal articular chondrocytes [Brenner et al., 1993]. Interestingly, in the present study bFGF proved to be the most potent growth factor. To the best of our knowledge this is the first report on the proliferative action of bFGF on human fetal chondrocytes. Thus the results provide the basis to understand the pathogenesis of growth deficiency in those cases of achondroplasia, hypochondroplasia and TD where mutations in the FGFR3 have been identified.

TABLE IV. Clonal Growth of Articular Chondrocytes From Fetal Controls and Patients With Thanatophoric Dysplasia (TD) in Response to IGF-I (% of Basal Value)

IGF-I	0.3 ng/ml (%)	1.25 ng/ml (%)	12.5 ng/ml (%)
Controls (M ± SD, n = 5)	127 ± 18	161 ± 19	172 ± 48
Range	106–147	147–191	118–247
TD 1[a]	120 ± 24	132 ± 14	157 ± 23
TD 2[a]	100 ± 4	113 ± 4	120 ± 8
TD 3[a]	126 ± 22	148 ± 33	171 ± 15
TD 4[a]	100 ± 4	145 ± 12	155 ± 14
TD 5[a]	131 ± 18	135 ± 8	130 ± 15

[a]M ± SD of triplicate determinations.

TABLE V. Conal Growth of Articular Chondrocytes From Fetal Controls and Patients With Thanatophoric Dysplasia (TD) in Response to IGF-II (% of Basal Value)

IGF-II	0.3 ng/ml (%)	1.25 ng/ml (%)	12.5 ng/ml (%)
Controls (M ± SD, n = 5)	178 ± 54	188 ± 60	193 ± 72
Range	123–256	136–278	140–313
TD 1[a]	131 ± 18	135 ± 17	177 ± 18
TD 2[a]	101 ± 3	105 ± 5	125 ± 6
TD 3[a]	157 ± 7	181 ± 23	167 ± 11
TD 4[a]	111 ± 5	140 ± 8	144 ± 12
TD 5[a]	134 ± 8	142 ± 11	126 ± 5

[a] M ± SD of triplicate determinations.

In our cases of TD type I the proliferative response of articular chondrocytes to TGF-β1 which acts via a serine/threonine kinase receptor [Massague, 1992] was not impaired. In contrast, the response to bFGF and IGF-I/II was heterogenous. Chondrocytes of one individual patient did not respond normally to bFGF, while the action of the other growth factors tested was not markedly disturbed. In this case a mutation of the FGFR3 may be a good candidate for the primary defect, since this type of FGF receptor is predominantly expressed in cartilage and brain during fetal development [Peters et al., 1993]. In another patient the response to both bFGF and IGF-I/II was reduced. IGF I and II exert their proliferative effect both via the type I IGF receptor [Brenner et al., 1993]. Therefore, the concordant result for these two growth factors is adequately well explainable. Since FGF-receptors and the type I IGF receptor both are tyrosine kinase receptors [Ullrich and Schlessinger, 1990] there might be some synergistic action that could be disturbed by defects in one of these receptors.

In a third TD fetus the response to bFGF was only slightly below the range of controls and it is not known so far whether this observation has any relation to the underlying defect. In this case the other factors tested as well were within the normal stimulation limits. In the remaining two cases the responses to the growth factors tested were not impaired, so that a receptor defect for bFGF, IGF-I/II, and TGF-β1 is unlikely.

A comparison of our results with clinical, radiologic and histologic data of the patients indicated that the case in which only the effect of bFGF was reduced had several distinct aspects. It was the only case in which there was no bell-shaped thorax, only a slightly reduced proliferative and hypertrophic zone of the growth plate and no increased periosteal ossification. However, it was the youngest patient studied and these differences may be simply explained by the influence of gestational age. Therefore, despite heterogenous in vitro results of the chondrocyte proliferation data, no distinct radiological and histological finding could be clearly attributed to patients with low or normal response to bFGF and/or IGF-I/II.

In conclusion, our study on the clonal growth of articular chondrocytes demonstrated defects in the regulation of chondrocyte proliferation in some cases with TD. They could well account for the severely reduced proliferation zone of the growth plate. We also present

TABLE VI. Clonal Growth of Articular Chondrocytes From Fetal Controls and Patients With Thanatophoric Dysplasia (TD) in Response to TGF-β1 (% of Basal Value)

TGF-β1	0.3 ng/ml (%)	1.25 ng/ml (%)
Controls (M ± SD, n = 5)	166 ± 34	188 ± 35
Range	134–224	126–208
TD 1[a]	196 ± 4	203 ± 15
TD 2[a]	143 ± 13	143 ± 13
TD 3[a]	214 ± 65	221 ± 13
TD 4[a]	192 ± 9	244 ± 3
TD 5[a]	155 ± 6	211 ± 11

[a] M ± SD of triplicate determinations.

cell-biological evidence that TD may be caused by heterogenous molecular defects. Further studies may elucidate the exact nature of the underlying defects in signal transduction and provide new information not only for the pathogenesis of TD but also for the normal regulation of human fetal growth.

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